# Role of GLTSCR2 in the regulation of telomerase activity and chromosome stability

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**Abstract.** Telomerase is essential for regulating telomeres, and its activation is a critical step in cellular immortalization and tumorigenesis. The transcriptional activation of human telomerase reverse transcriptase (hTERT) is critical for telomerase expression. Although several transcriptional activators have been identified, factors responsible for enhancing the hTERT promoter remain to be fully elucidated. In the present study, the role of glioma tumor-suppressor candidate region gene 2 (GLTSCR2) in telomerase regulation was analyzed. A doxycyclin-inducible green fluorescent protein (GFP)-tagged GLTSCR2-expressing adenovirus (Ad-GLT/GFP) was used for the transduction of SK-Hep-1 and T98G cancer cells, and normal human umbilical vein endothelial cells. Changes in telomerase activity using telomere repeat amplification protocol assay were assessed, and the gene expression levels of hTERT were then examined. To investigate chromosome instability and senescence, Giemsa and β-galactosidase staining was performed. The results revealed that overexpression of GLTSCR2 significantly increased telomerase activity in the cancer and normal cell lines. This increase was consistent with increases in the protein and mRNA expression levels of hTERT. In luciferase assays, the hTERT promoter was activated by GLTSCR2. Knockdown of GLTSCR2 led to the downregulation of telomerase activity, abnormal nuclear morphology as a marker of chromosome instability, significant suppression of growth rate, alterations in cellular morphology and, eventually, cellular senescence. Taken together, the results of the present study suggested that GLTSCR2 is crucially involved in the positive regulation of telomerase and chromosome stability.

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## Introduction

Telomerase is essential for maintaining telomeres, and consists of an RNA template and a catalytic protein subunit, telomerase reverse transcriptase (TERT), which adds a six-base DNA repeat sequence (TTAGGG) to the telomere (1). With decreasing telomerase activity, the ends of chromosomes become shortened as somatic cells age or mature (2,3). Extensive efforts have been made to clarify the molecular mechanisms underlying telomerase activation. Human (h) TERT is a critical determinant of telomerase activation within the cell (4,5). Although a number of transcription factors have been identified as regulators of the hTERT promoter (6), the specific factors responsible for determining telomerase activity in cells remain to be fully elucidated.

Previously, the nucleolar protein, GLTSCR2, was shown to be a putative tumor suppressor gene, as it is capable of inducing phosphatase and tensin homolog-dependent apoptotic cell death and inhibiting tumor growth (7-9). However, the biological function and molecular mechanisms of GLTSCR2 remain to be fully elucidated. In our previous study, it was reported that GLTSCR2 functions as a DNA damage response protein in the ataxia telangiectasia mutated (ATM)-Chk2 and ataxia telangiectasia and Rad3-related protein (ATR)-Chk1 pathways (10). Notably, increasing evidence indicates that several DNA damage response proteins are involved in telomere maintenance. Mutations in proteins involved in the response to DNA damage result in telomere dysfunction and subsequent chromosomal instability, suggesting extensive functional interactions between telomere maintenance and DNA damage response mechanisms (11-14). In the present study, the effect of GLTSCR2 on telomerase activity in various cell types was examined. Subsequent to this, the effect of the knockdown of GLTSCR2 on cellular morphology and growth rate was investigated. The present study revealed a novel biological function of GLTSCR2 in maintaining chromosomal stability via telomerase regulation.

## Materials and methods

*Cell culture and treatment*. The SK-Hep-1 cells, T98G cells and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in a humidified

5% $CO_2$ incubator maintained at 37˚C, either in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; for SK-Hep-1 and T98G cells) or in endothelial growth medium-2 (HUVECs; Thermo Fisher Scientific, Inc) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc).

*Antibodies and reagents*. The anti-GLTSCR2 polyclonal antibody was produced by the present group by immunizing a rabbit with keyhole limpet hemocyanin-conjugated amino-acid residues 78-193 (CTRAKPGPQDTVERPF) of human GLTSCR2. The antibody was purified from the immune serum by affinity chromatography (8). The rabbit polyclonal anti-human TERT (1:1,000; SC-7212) and the rabbit polyclonal anti-tubulin antibodies (1:1,000; SC-9104) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

*Construction of the Tet-Off adenoviral-mediated system*. A GFP-GLTSCR2 construct consisting of full-length GLTSCR2 cDNA fused to a C-terminal GFP tag was first cloned into pTRE-Shuttle2 vector (Clontech Laboratories, Inc., Mountain View, CA, USA), which contains the tetracyclineresponsive element (TRE) upstream of the CMV minimal promoter. The resulting TRE-GFP-GLTSCR2 expression unit was excised from the pTRE-Shuttle2 vector using I-Ceu I and PI-Sce I resection enzymes, and then ligated to Swa I-digested Adeno-X System 1 Viral DNA (Clontech). The resulting recombinant Adeno-XGFP-GLTSCR2 vector (Ad-GFP/GLT) was packaged into infectious adenoviral particles by transfection of HEK293 cells, and recombinant adenovirus was harvested by lysing transfected cells. In the Tet-off system, the tet-responsive transcriptional activator is expressed and binds to the TRE in the absence of doxycycline (Clontech; stored in aliquots and kept at -20˚C) to activate transcription of GLTSCR2. As doxycycline is added to the culture medium at 37˚C, transcription from the TRE is turned off in a dose dependent manner. To transiently express GLTSCR2, the cells were plated in a 6-well plate at 70% confluence ($5x10^5$ cells) and coinfected with a recombinant adenovirus, [Ad-green fluorescent protein (GFP)/GLT], and a regulation virus (Adeno-X Tet-Off virus) with a multiplicity of infection (MOI) of 100 in serum-free media for 12 h. Fresh complete medium was subsequently added.

*Generation of stable GLTSCR2-knockdown cell lines*. For knockdown of the expression of GLTSCR2, feline leukemia virus-based lentiviral GLTSCR2 shRNA vectors were purchased from GeneCopoeia, Inc. (Rockville, MD, USA). The GLTSCR2-target sequence was 5'-GAGACCGGTTCA AGAGCTT-3', and the scrambled (Scr) sequence was 5'-CGA TACTGAACGAATC-3'. Cells were seeded at $5x10^5$ cells in 30 mm dishes and lentiviral stocks of the GLTSCR2 shRNA or the control shRNA vector were then incubated with separate sets of cells with an MOI of 10. After 48 h, clones of GLTSCR2-knockdown cells were selected for using puromycin (1 $\mu$g/ml; Sigma-Aldrich) treatment. Protein expression levels were analyzed using western blot and immunocytochemical analyses.

*Telomere repeat amplification protocol (TRAP)*. A TRAP assay was used to detect telomerase activity, in which a TRAPEZE® XL telomerase detection kit (EMD Millipore, Billerica, MA, USA) was used, according to the manufacturer's protocol. Briefly, cell pellets were resuspended in 200 $\mu$l of 3-[cholamidoprophyl)-dimethylammonio]-1-propane-sulfonate lysis buffer and incubated for 30 min on ice. After centrifugation at 13,000 g for 20 min at 4˚C, aliquots of the supernatant were rapidly frozen and store -80˚C (15). The telomeric DNA from the cell extracts was amplified by 36 cycles of polymerase chain reaction (PCR) with a denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec, and elongation at 72˚C for 1 min. 20 $\mu$l of the PCR product were subjected to electrophoresis on a non-denaturing 10% polyacrylamide gel (Sigma-Aldrich) The experiment was repeated four times.

*Reverse transcription semi-quantitative PCR (RT-PCR) analysis*. Total RNA (1 $\mu$g) was extracted using Trizol reagent (Invitrogen) from the cultured cells and converted into cDNA using a cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. hTERT and tubulin mRNA amplification were performed with the following primers: hTERT forward, 5'-AGAGTGTCTGGAGCAAGTTGC-3', hTERT reverse, 5'-CGTAGTCCATGTTCACAATCG-3'; tubulin forward, 5'-CATGTATCTTCCATACCCTG-3', tubulin reverse, 5'-CTGAAGGTATTCATGATGCG-3'. The cycling conditions were as follows: Denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, elongation at 72˚C for 30 sec. The resulting PCR products were separated on 2% agarose gels (Sigma-Aldrich) and visualized using ethidium bromide staining (0.5 $\mu$g/ml; Sigma-Aldrich). The quantification of the transcriptional gene expression was performed using the Gel Doc EZ system Image Lab™ software (Bio-Rad Laboratories, Inc., Richmond, CA, USA) and normalized to the expression of tubulin as an endogeneous control. The method used for quantification was semi-quantitative evaluation using densitometric analysis (16)

*Luciferase assay*. The pGL2 luciferase plasmid (Promega Corporation, Madison, WI, USA) was used. The hTERT promoter sequence was amplified by PCR to generate a 1580 bp fragment from human genomic DNA using upstream primers (5'-AGCGATACCTATTGAATGCC-3') containing a *Kpn*I site, together with a single downstream primer (5'-TCTCCTCGCGGCGCGAGTTT-3') containing a *Hin*dIII site. Luciferase activity was measured in samples containing equivalent quantities of protein using a luminometer and luciferase assay reagents (Promega Corporation). The protein content of cell lysates was determined using BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL, USA) in clear 96-well plates (Nunc, Wiesbaden, Germany), and colorimetric assay was performed using an VersaMax ELISA Microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The equivalent quantities of protein were used in the subsequent luciferase activity assay.

*Giemsa staining and slide preparation*. Fixed slides were immersed in 5% Giemsa (Sigma-Aldrich) for 20 min. The slides were then rinsed briefly in $dH_2O$ and air-dried. For

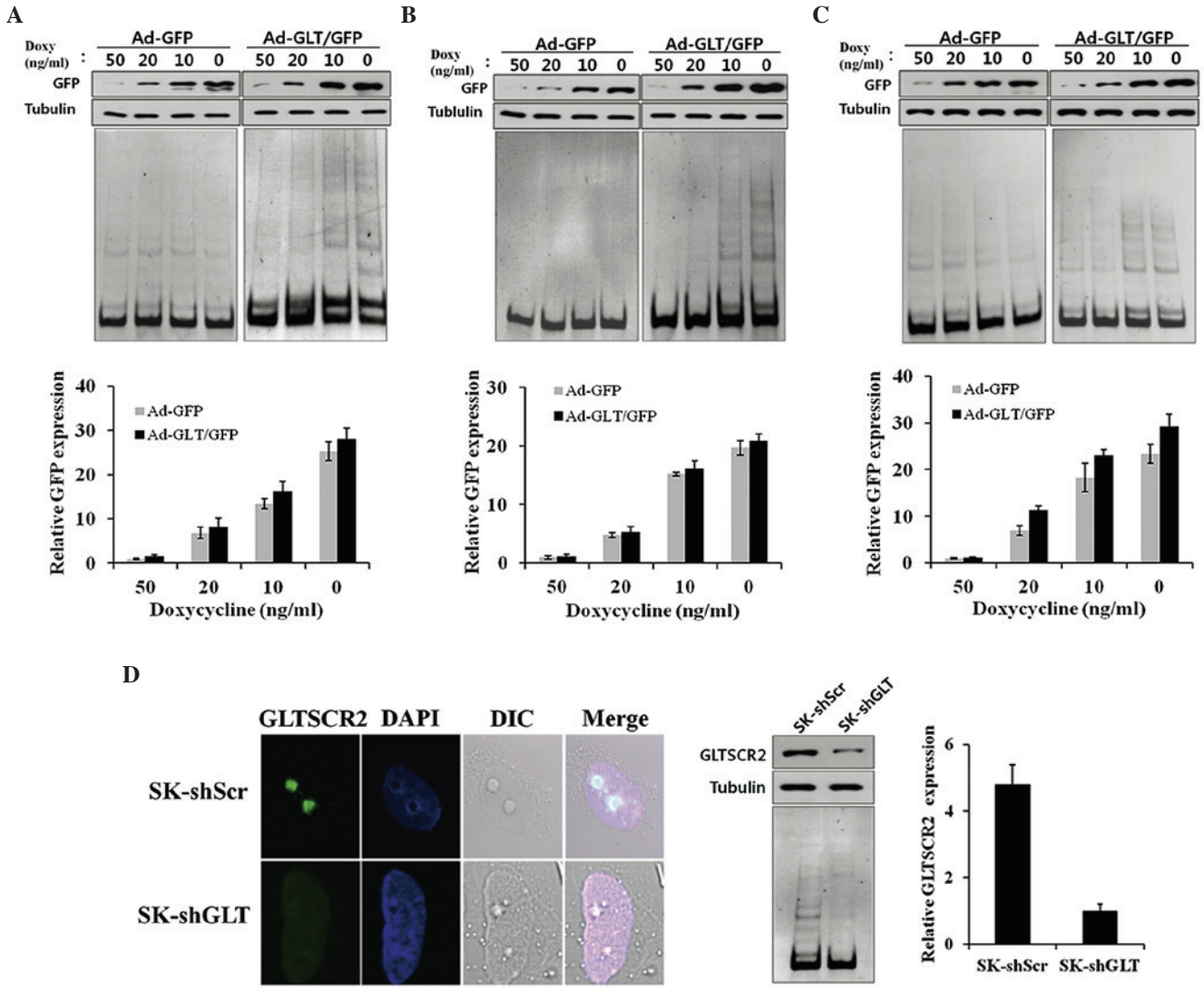


Figure 1. GLTSCR2 increases telomerase activity. (A) SK-Hep-1 cells, (B) T98 G cells and (C) human umbilical vein endothelial cells were infected with Ad-GFP or Ad-GLT/GFP, and incubated with decreasing concentrations of doxycycline (50, 20, 10 and 0 ng/ml). (D) SK-Hep-1 cells were stably infected with lentivirus carrying either GLTSCR2-targeting SK-shGLT or SK-shScr, immunostained with anti-GLTSCR2 and DAPI, and viewed under an DIC-equipped confocal microscope (Left, magnification x630). Cells were harvested, and lysates were examined using western blotting with the indicated antibodies. Tubulin was used as a loading control. TRAP assays were performed using subconfluent proliferating cultures of cells. GLT, glioma tumor-supressor; GLTSCR2, glioma tumor-suppressor candidate region gene 2; GFP, green fluorescent protein; sh, short hairpin RNA; Scr, scambled; Doxy, doxycycline.

long-term storage, the slides were mounted with a drop of Permount (Thermo Fisher Scientific, Inc.) and coverslips.

*Senescence-associated β-galactosidase staining*. The cells were fixed with 2% formaldehyde/0.2% glutaraldehyde solution (both Cell Signaling Technology, Inc., Danvers, MA, USA) and stained overnight at 37˚C with 1 mg/ml X-gal (Cell Signaling Technology, Inc.), 40 mM citric acid/$Na_2HPO_4$ (pH 6.0; Sigma-Aldrich), 5 mM potassium ferrocyanide/ferricyanide (Cell Signaling Technology, Inc.), 150 mM NaCl and 2 mM $MgCl_2$, both Sigma-Aldrich.

*Statistical analysis*. In all cases, the results are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). A two-tailed, unpaired Student's *t*-test was used for data analysis. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

To determine the effect of GLTSCR2 on telomerase activity, the present study first measured the level of telomerase activity in the cultured SK-Hep-1 cells. Initially, the SK-Hep-1 cells were infected with a doxycycline-inducible (Tet-Off system) adenovirus, expressing either GFP-tagged GLTSCR2 (Ad-GLT/GFP) or GFP (Ad-GFP) as a control, and placed in media containing different concentrations of doxycycline (0, 10, 20 and 50 ng/ml) for 48 h. Telomerase activity was measured using a TRAP assay, as described above. As shown in Fig. 1A, the Ad-GLT/GFP cells showed significantly increased telomerase activity in proportion to the expression levels of GLTSCR2, compared with the Ad-GFP cells. The effects of GLTSCR2 in the T98G glioblastoma cells were similar to those observed in the SK-Hep-1 cells (Fig. 1B).

The majority of cancer cells express high levels of telomerase activity. However, human primary normal cells usually

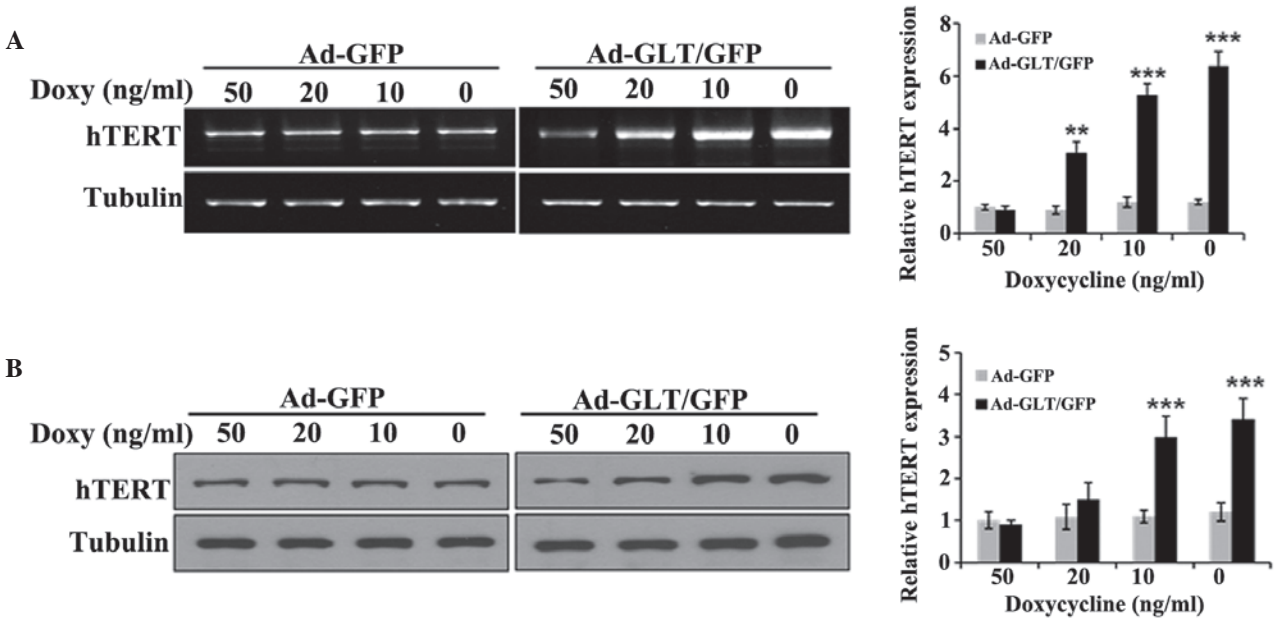


Figure 2. Overexpression of GLTSCR2 causes upregulation of the mRNA and protein expression of hTERT. SK-Hep-1 cells were infected by Ad-GFP or Ad-GLT/GFP. The cells were then harvested to isolate RNA for (A) semi-quantitative RT-PCR assays or to prepare whole-cell lysates for (B) western blotting The expression level of hTERT in the GLTSCR2-overexpressing Ad-GFP/GLT cells was significantly higher than those in the corresponding control (Ad-GFP) cells. Expression levels were quantified by densitometric analysis of banding intensity. Data are presented as the mean ± standard deviation of three independent experiments. $^{**}$P<0.01, $^{***}$P<0.001 vs. Doxy 50 ng/ml. hTERT, human telomerase reverse transcriptase; GLT, glioma tumor-supressor; GLTSCR2, glioma tumor-suppressor candidate region gene 2; GFP, green fluorescent protein; Doxy, doxycycline.

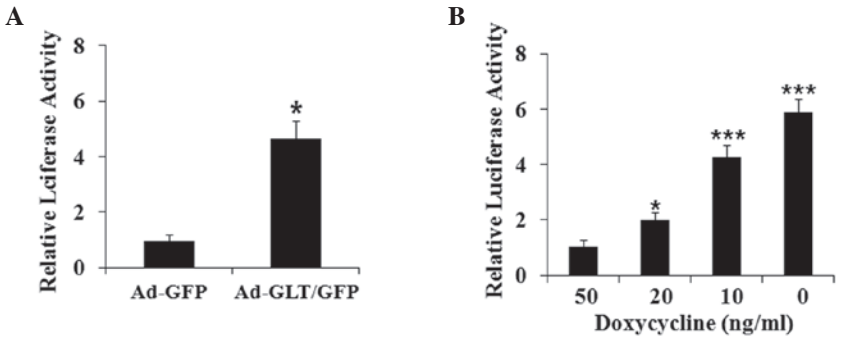


Figure 3. GLTSCR2 increases the transcriptional activity of the hTERT promoter. (A) SK-Hep-1 cells were transfected with the hTERT promoter plasmid using Lipofectamine for 24 h, and then infected with Ad-GFP or Ad-GLT/GFP. Following another 24 h incubation, the cell lysates were extracted, and luciferase assays were performed. hTERT promoter activity was increased in the GLTSCR2-overexpressing cells $^{***}$P<0.001 vs. Ad-GFP. (B) SK-Hep-1 cells were infected with Ad-GFP or Ad-GLT/GFP with decreasing concentrations of doxycycline (50, 20, 10 and 0 ng/ml, respectively). hTERT promoter activity was induced dose-dependently by GLTSCR2. Data are presented as the mean ± standard deviation of three independent experiments. $^{*}$P<0.05, $^{***}$P<0.001 vs. Doxy 50 ng/ml. hTERT, human telomerase reverse transcriptase; Doxy, doxycycline.

exhibit a low level of telomerase activity (17,18). If GLTSCR2 is an inducer of telomerase activity, its expression may induce telomerase activity in primary normal cells. Thus, the present study examined the effect of GLTSCR on telomerase activity in HUVECs. The HUVECs were infected with Ad-GLT/GFP or Ad-GFP for 48 h, and the TRAP assay was performed. Telomerase activity was low and almost undetectable in the Ad-GFP HUVEC cells. However, telomerase activity in the Ad-GLT/GFP HUVEC cells was significantly increased and correlated with the expression level of GLTSCR2, which was similar to the results observed in the SK-Hep-1 and T98G cells (Fig. 1C).

To further demonstrate the effect of GLTSCR2 on telomerase activity, the SK-Hep-1 cells were stably infected with a lentivirus carrying either GLTSCR2-targeting shGLT or shScr. Subsequent immunofluoresence and immunoblotting confirmed that the expression of GLTSCR2 was significantly reduced, by >80%, in the cells stably infected with shGLT (Fig. 1D). In contrast to the GLTSCR2-overexpressing SK-Hep-1 cells, the SK-shGLT cells showed lower telomerase activity, compared with the control (SK-shScr) cells (Fig. 1D). These results suggested that GLTSCR2 exerted a positive effect on telomerase activity.

To investigate the mechanism by which GLTSCR2 increases telomerase activity, the present study examined whether GLTSCR2 affected the expression of hTERT, a catalytic subunit of telomerase and a critical determinant of telomerase activity. The SK-Hep-1 cells were infected with

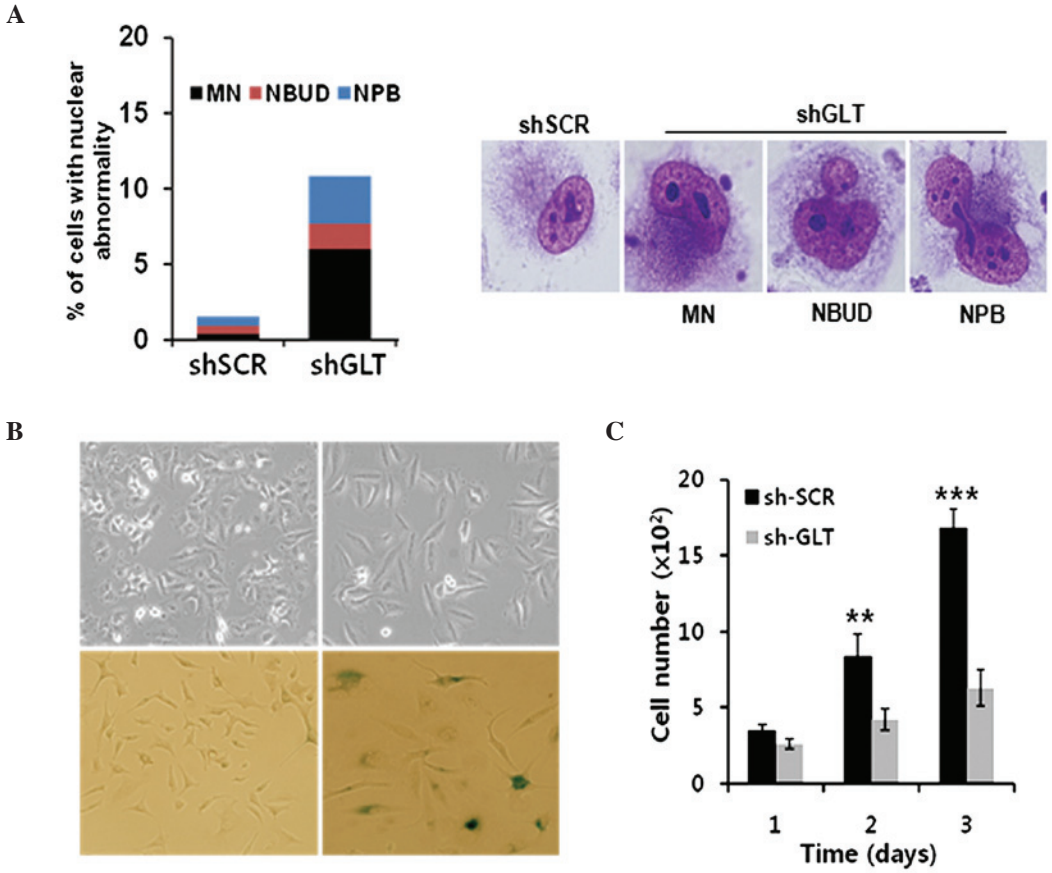


Figure 4. Knockdown of GLTSCR2 induces abnormal nuclear morphology and cellular senescence. The T98G cells were stably infected with a lentivirus carrying either GLTSCR2-targeting shGLT or scrambled shRNA. (A) T98G-shGLT cells showed nuclear abnormalities, visualized by staining with Giemsa, including MN, NBUD and NPB, magnification x630. A minimum of 300 cells were counted and the count was performed three times. (B) Top and bottom panels show morphological changes and cellular senescence by β-galatosidase staining, in the T98G-shScr and T98G-shGLT groups. Magnification, x100. (C) Growth rates of the cells were determined by total cell count at the time points indicated. Data are presented as the mean ± standard deviation of three independent experiment. sh, short hairpin RNA; GLT, glioma tumor-supressor; GLTSCR2, glioma tumor-suppressor candidate region gene 2; Scr, scambled; MN, micronucleus; NBUD, nuclear buds; NPB. nucleoplasmic bridges. $^{**}$P<0.01, $^{***}$P<0.001 vs. shSCR 1 day.

Ad-GLT/GFP or Ad-GFP for 48 h. The mRNA and protein expression levels of hTERT were then evaluated using RT-PCR analysis and western blotting, respectively. Compared with the Ad-GFP control cells, the Ad-GLT/GFP cells exhibited increased mRNA and protein levels of hTERT (Fig. 2A and B).

To investigate the mechanism underlying the GLTSCR2-induced expression of hTERT, the present study examined the effect of GLTSCR2 on the activity of the hTERT gene promoter by using the hTERT promoter-luciferase reporter plasmid, pGL2-hTERT, in the SK-Hep-1 cells. The SK-Hep-1 cells were transiently transfected for 24 h with pGL2-hTERT, and were then infected with either Ad-GLT/GFP or Ad-GFP for 24 h to allow for the expression of GLTSCR2. As shown in Fig. 3A, Ad-GLT/GFP markedly induced hTERT promoter activity, whereas Ad-GFP had minimal or no effect on hTERT promoter activity. The induction of pGL2-hTERT activity by GLTSCR2 was dose-dependent (Fig. 3B). Together, these data demonstrated consistent positive regulation of the hTERT gene by GLTSCR2 in the SK-Hep-1 cells.

Telomeres are essential for maintaining chromosome stability, and extensive telomere shortening can lead to abnormal nuclear morphologies, including micronuclei, nucleoplasmic bridges or nuclear buds as a marker of chromosome instability (19). Subsequently, the effect of the knockdown of GLTSCR2 on abnormal nuclear morphology was examined. The presence of micronuclei, nucleoplasmic bridges and nuclear buds increased in the T98G-shGLT cells (Fig. 4A).

The present study also examined the effects of the knockdown of GLTSCR2 on cellular morphology and growth rate. The T98G-shGLT cells presented with significant morphological changes, characterized by an elongated and enlarged flat shape, resembling the morphology of senescent cells, compared with parental T98 G cells (Fig. 4B). The growth rate was reduced in the T98G-shGLT cells (Fig. 4C). Additionally, the T98G-shGLT cells entered cellular senescence, as determined by senescence-associated β-galactosidase staining (Fig. 4B). Taken together, these results suggested that GLTSCR2 is crucially involved in the positive regulation of telomerase and chromosome stability.

## Discussion

In the present study, to investigate the effect of GLTSCR2 on telomerase activity, human SK-Hep-1 and T98G cancer cells, and normal HUVECs were infected with Ad-GLT/GFP in a doxycycline-inducible (Tet-Off system) manner. Subsequently, changes in telomerase activity and the expression of hTERT were determined. GLTSCR2 significantly increased telomerase

activity in the cancer and normal cells. This increase was consistent with an increase in the expression pf hTERT, as determined by semi-quantitative RT-PCR analysis, western blot analysis and promoter assays for hTERT. Although the molecular mechanism underlying the regulation of telomerase activity by GLTSCR2 remains to be fully elucidated and currently under investigation, these results suggested that GLTSCR2 is crucially involved in the positive regulation of telomerase maintenance.

Several other studies have been performed to identify the transcriptional factors critical for hTERT regulation (20-22), however, no specific factors have been identified, which are responsible for determining telomerase activity in cells. It is now widely accepted that a number of nuclear factors are involved in the regulation of hTERT transcription(23,24). Furthermore, hTERT undergoes a regulated subnuclear translocation between the nucleoplasm and the nucleolus (25). hTERT nucleolar transportation may be an essential step for the biogenesis of telomerase in human cells (26,27). The telomere-capping and stabilizing proteins, telomeric repeat binding factor (TRF)1 and TRF2, also accumulate in the nucleolus, and they are modulated by nucleolar proteins (28). Within these complex regulatory mechanisms, GLTSCR2 appears to be involved. The present study suggested that the GLTSCR2 nucleolar protein is a crucial determinant of telomerase activity in normal and cancer cells.

Human primary cells usually exhibit low levels of telomerase activity. However, the majority of cancer cell lines express high levels of telomerase activity, which may support continued cell proliferation (17,18). In GLTSCR2-overexpressing SK-Hep-1 cells and control cells, no significant differences in cell proliferation were observed (data not shown), although telomerase activity was significantly increased in the former. By contrast, in the GLTSCR2-knockdown cells, cell proliferation was significantly lower, compared with that in the control cells. These results indicated that it is unlikely that cell proliferation contributed to the increased telomerase activity observed in the GLTSCR2-overexpressing cell lines. However, in the GLTSCR2-knockdown cells, decreased telomerase activity may have impeded proliferation. Thus, GLTSCR2, but not the rate of cell proliferation, appears to be important in telomerase activity.

Reports have indicated that the cellular DNA damage response (DDR) ensures genomic stability and protects against genotoxic stresses. By contrast, defects in the DDR contribute to genomic instability (10). For example, defects in ATM, a key DNA damage signaling molecule, are associated with telomere loss, telomeric fusions and extrachromosomal telomeric DNA appearance in cells from patients with ataxia telangiectasia and ATM-deficient mice (12). In addition, the TRF1 and TRF2 proteins, which stabilize telomeres, are known to be important in the DDR. In particular, the inhibition of the DDR by dominant negative alleles or by the genetic deletion of TRF2 in mouse cells gives rise to a potent checkpoint response and frequent end-to-end fusions (29). Furthermore, TRF2 is rapidly phosphorylated in response to DNA damage, likely via an ATM-kinase-mediated pathway, which is critical for telomere maintenance (12,30). Upon phosphorylation, TRF2 rapidly localizes to sites of DNA damage, specifically double-strand breaks, where it acts as an early component of the DNA repair response system (14). GLTSCR2 mediates the activation of ATM-Chk2 and ATR-Chk1 in response to DNA damage (10). In the present study, it was shown that the siRNA-based knockdown of GLTSCR2 led to abnormal nuclear morphology, including the presence of micronuclei, nucleoplasmic bridges and nuclear buds as a indicator of chromosome instability, which indicated an impaired response of the cells to DNA damage.

In conclusion, the results of the present study suggested that GLTSCR2 is a crucially involved in the positive regulation of telomerase and chromosome stability. The precise role of GLTSCR2 in the regulation of telomerase remains to be elucidated. However, GLTSCR2-induced telomerase regulation may provide important clues regarding the role of GLTSCR2 in chromosome stability. Continued investigations into other GLTSCR2-associated proteins involved in the DDR are critical to determine the extent of overlap between telomere maintenance and DNA repair response signaling pathways.

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